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Immobilization Parameters Statistically Optimized for Whole Cells of *Pseudomonas putida* G7 to Enhance Limonin Biotransformation

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Abstract: This study was aimed for optimizing the immobilization parameters for *Pseudomonas putida* G7 in Ca-alginate beads, in order to establish a debittering strategy for citrus juices, by biotransforming the bitter principle - Limonin. Response Surface Methodology (RSM) with Central Composite Design (CCD) was employed to model the significant parameters for an enhanced response. An enhanced limonin bioconversion and immobilized bead stability was obtained with alginate concentration (2%), cell load (47.2g/l), and a bead diameter (2.1mm); which had significant effects ($p < 0.001$) on limonin biotransformation. The R^2 values of 0.9 showed good agreement between experimental and predicted response. Validation experiments under optimized parameters showed good association between experimental (limonin biotransformation and stability response of 65.8% and 0.97 OD respectively) and predicted responses (limonin biotransformation and stability of 65.1% and 0.094 respectively). Thus, the approach is promising to develop a strategy for debittering citrus juices by biotransforming limonin at a faster rate.

Keywords: Response Surface Methodology, Central Composite Design, Na-alginate, Cell load, Bead diameter.

1. Introduction

Immobilized biocatalysts - whole microbial cells (viable or nonviable) or their enzymes have some clear advantages over employing free cells in bioprocesses[1,2]. Cell immobilization provides a physical incarceration or localization of microbial cells or enzymes to a certain defined space for the preservation of certain desired catalytic activity and subsequent continuous operation stability[3]. Industrial application of immobilized enzymes has been limited due to several factors like low stability, low recovery, low yield and expensive steps involved in the isolation and purification of enzymes. Therefore, whole cell immobilization has been employed and offers many advantages over immobilized or free enzymes and free microbial cells. This method is cost effective, offers continuous operational stability and carry out multi-step cofactor requiring bioconversion[4,5] with minimum downstream processing, decreased product inhibition, simplified biocatalyst recovery, high re-usability of cells, relative ease of product separation, high volumetric productivity and reduced susceptibility of cells to contamination[6]. Microbial cells and their enzymes (both in free form and immobilized forms) have been employed for the development of strategies for the eradication of bitterness from the citrus juices[7,8]. Among the various limonoids in citrus juices, limonin – a triterpenoid dilactone is a major cause which imparts bitterness to the citrus juices and is a hindrance for the citrus industries worldwide, in terms of bitterness and "delayed bitterness"[7,9,10]. The negative impact created by a bitter taste in citrus juices made debittering a generally incorporated step in industrial juice processing technology. Previous studies explaining the usage of various immobilizing matrixes (for immobilizing various limonin degrading microbes for debittering citrus juices) like polyacrylamide gel, k-carrageenan and polyurethane foam has faced a number of constraints like limitations for human consumption, low stability matrix at low pH of juice and delayed reduction of bitterness in citrus juices[11].

Various immobilization materials and techniques are being developed, making the immobilized biocatalysts more reachable for industrial applications. Entrapment technique has proved to be the most

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significant of all immobilization techniques, for immobilizing whole cells into gel matrices[12]. Entrapment of living microbial cells in the gel matrix of alginate has proved to be quite promising and has been widely used and the technique is simple, fast, cost effective, biocompatible, chemically inert, functional in the organic phase or biphasic system[13-15]. However, immobilized system faces a limitation of diffusional resistances to both substrate and product formed, which in turn determine the overall yield or biotransformation. Various physiological factors such as alginate concentration, cell loading and bead diameter are considerable parameters for immobilization[16]. Therefore, optimization of such parameters is an important prerequisite.

Response surface methodology (RSM) is a collection of statistical technique that has been applied successfully in biotechnology, for optimization and evaluation of significant parameters for desired responses in various processes[17,18]. The conventional approach is time consuming and involves variation of one variable at a time, and limits the evaluation of the combined effects of all the factors involved in the process. Based on the multivariate non-linear model (which reduces the number of experiments, improves statistical interpretation possibilities, reduction in number of experiments and time required for overall analysis), RSM is useful for the evaluation and better understanding of the interactions of the various significant parameters within a limited number of experiments[19,20].

The objective of this study was to employ response surface methodology for the best optimization of immobilization parameters (alginate concentration, cell load, and bead diameter) and to detect their interaction and combined effects to obtain maximum response (activity and stability).

2. Materials and Methods

2.1 Microorganism and Culture Conditions

The lyophilized bacterial strain of *P. putida* G7 (MTCC 1658) was procured from the microbial type culture collection and a gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The nutrient medium for *P. putida* G7 was Luria-Bertani broth and mineral minimal medium (M63) supplemented with 0.5% limonin. The strain was grown with shaking at 150 RPM at 30°C. The log phase cultures were used for agar and calcium alginate immobilization. All chemicals were purchased from Merck (Darmstadt, Germany), except Na-alginate and agar, which were purchased from Sigma (St. Louis, MO, USA).

2.2 Immobilization of whole cells

The limitation and low response of immobilization by adsorption and attachment[21,22] as compared to entrapment method, has helped to choose the entrapment method for this study. The bacterial cells of *P. putida* G7 were immobilized by entrapment using different matrices like agar and sodium alginate as explained by Tapingkae *et al*., (2010). A sterile standard buffer containing a range of 0.5% to 4% (w/v) of sodium alginate and agar (Sigma, St. Louis, MO, USA) were prepared by autoclaving it at 121°C (15 psi) for 15 min. Both the immobilizing solutions (Na-alginate and agar solution of variable concentrations) (2.5ml) at 50°C were mixed with 2.5ml of buffer containing 1g of wet weight of cell at 60°C. Sterile syringes (2ml) were then filled with the cell/alginate and cell/agar solutions and 0.5ml was dropped through a 0.45mm x 12mm needles into a 25ml of stirring 0.2M chilled $CaCl_2$ solution respectively. Another set of beads was prepared with Na-alginate and agar which does not harbor any bacterial cells and were taken as controls. Each set of beads was transferred to sterile tubes and were kept on shaking condition in a 0.3M $CaCl_2$ solution overnight at 4°C.

Several different cell loads of 5, 10, 20, 30, 40, 50, 60mg/ml were also used for immobilization with variable bead diameters of 1-4mm, during the maximum limonin biotransformation in citrus juice using immobilized cells.

2.3 Limonin biotransformation in citrus juices using immobilized cells of *P. putida* G7

The log phase revived culture of *P. putida* G7 with variable cell loads were used in immobilized forms (agar and Na-alginate), for limonin biotransformation in the citrus juice (100ml), taken in an Erlenmeyer flask and incubated at 30ºC. The regular aliquots of the juice sample from all the sets of free and immobilized (agar and Na-alginate) were aseptically withdrawn at regular intervals of one hour, till 36 hours. The aliquots were centrifuged at 10,000 RPM for 2 minutes at 4ºC. The pellet was then resuspended in 1ml of saline (0.85% NaCl) and the optical density was recorded at a wavelength of 600nm.

2.4 Estimation of cell mass and viability

Cell growth was determined spectrophotometrically at 600nm and converted to cell count using a conversion factor (one unit at 600nm was equivalent to 10^5 CFU/ml, which corresponded to 28mg protein that was calculated by plotting standard graphs). In case of all immobilized conditions (agar and Na-alginate), 5 beads were dissolved in 2ml of phosphate buffer (pH 7.0) and cells were collected by centrifugation at 5000 rpm at 37°C and were used for cell mass measurement, by serial dilution plate count method on Luria agar plates and incubated at 37°C for 24 h. At the end of each batch, the cell densities in the beads were enumerated using similar method to study the total cell loss upon repeated use. Viable cell counts

were performed in duplicates and expressed in CFU/ml of immobilized beads.

2.5 Estimation of limonin biotransformation

The extraction and quantification of biotransformed limonin in the supernatant of the aliquots were done by estimating the residual limonin as explained by Vaks *et al*., (1981). One milliliter of the solution containing limonin was extracted with 2ml chloroform. The chloroform was evaporated to dryness and the residue was dissolved in 0.2ml of chloroform. The limonin content was measured by developing it with 2.5ml reagent (0.1g of 4-(dimethylamino) benzaldehyde; 3ml of acetic acid, and 2.4ml of 70% perchloric acid). The red color that has fully developed after 30 min. had a maximal absorbance at 503nm. The limonin in the unknown samples was estimated from a standard curve of pure limonin prepared similarly.

2.6 Selection of immobilisation method

Whole cells immobilised by entrapment in agar and Na-alginate were analyzed for the limonin biotransformation activity, protein loading ratio, and immobilization yield, which were defined as explained by Tapingkae *et al*., (2010) as follows:

$$\textbf{Biotransformation activity}\left(\frac{\textbf{units}}{\textbf{g}}\textbf{matrix}\right)=\frac{\textbf{Activity of immobilised whole cells (units)}}{\textbf{Actual weight of cell immobilised (g)}}$$

$$\textbf{Protein loading ratio}\ (\%)=\frac{\textbf{Amount of protein bound (mg)}}{\textbf{Amount of protein loaded (mg)}}\textbf{x100}\%$$

$$\textbf{Immobilisation yield}\ (\%)=\frac{\textbf{Total activity of immobilised whole cells (units)}}{\textbf{Total activity of free whole cells (units)}}\textbf{x100}\%$$

The immobilization method providing the maximal immobilization yield was chosen for further study.

2.7 Statistical optimization

After several preliminary tests based on one - factor experimental results, three critical parameters were selected which have a significant effect on whole cell immobilization. The Na-alginate concentration (X_1), Cell load (X_2) and bead diameter (X_3) were selected for further optimization. Response Surface Methodology (RSM) with Central Composite Design (CCD) was employed to detect the optimum levels and the interaction of significant variables. Table 1 represents the coded and non-coded values of the significant experimental variables at five coded (-α, -1, 0, +1, +α) levels. The zero levels of all the variables constitute to the central points while a combination of experimental variables consisting of one at its lowest level (-1) and its highest (+1) levels. A total of 20 experimental runs with different combinations of the significant parameters were carried out in triplicate that was important to estimate the response (Mean of the triplicates). The relationship of the significant parameters and their response (limonin biotransformation and bead stability) was calculated by a second order polynomial equation.

$$Y=\beta_0+\Sigma\,\beta_i\,x\,X_i+\Sigma\,\beta_{ii}\,x\,X_i^2+\Sigma\,\beta_{ij}\,x\,X_{ij}$$

Where Y is the predicted response, X_i is the independent variable, β_0 is the intercept term, β_i the linear effect, β_{ii} the squared effect and β_{ij} the interaction effect.

Design Expert software package (Version 2.05, Stat-Ease Inc., Minneapolis, USA) was used to estimate the response of dependent parameters and their optimum levels. Re-usability of the immobilized microbial cells and free cells were evaluated under the optimized levels of significant variables.

Table 1. Coded and non-coded values of the experimental variables

Variables	Coded Values				
	-α	-1	0	+1	+ α
A-Alginate concentration (%, w/v)	0.32	1	2	3	3.68
B-Cell load (mg/ml)	36.5	40	45	50	53.41
C-Bead diameter (mm)	1.16	1.5	2	2.5	2.84

3. Results and Discussion

3.1 Conventional studies

Among the various preliminary experiments performed for the selection of process parameters effecting limonin biotransformation in citrus juice, alginate concentration, cell load and bead diameter were screened as the most significant parameters for maximal limonin biotransformation by immobilized *P. putida* G7. Limonin in citrus juice was biotransformed up to 40% and 58% with *P. putida* G7 cells (cell load of 50mg/ml) immobilized in agar and alginate respectively (Fig. 1). Biotransformation response with cell immobilized in agar (concentration of 2.5% and bead diameter of 2.0mm) was lower i.e. 30%, as compared to the cells immobilized in alginate (concentration of 3% and bead diameter of 2.5mm) which showed a response of 57% in citrus juice (Fig. 2). Based on the response of conventional studies, alginate was preferred as a matrix for immobilizing *P. putida* G7 cells to direct the further studies for limonin biotransformation in citrus juice.

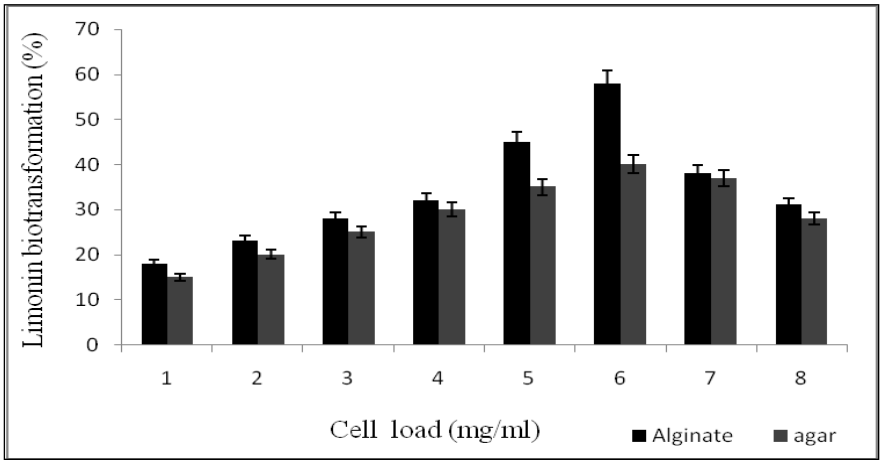


Fig. 1. Effect of cell load concentration (mg/ml) in alginate beads on limonin biotransformation.

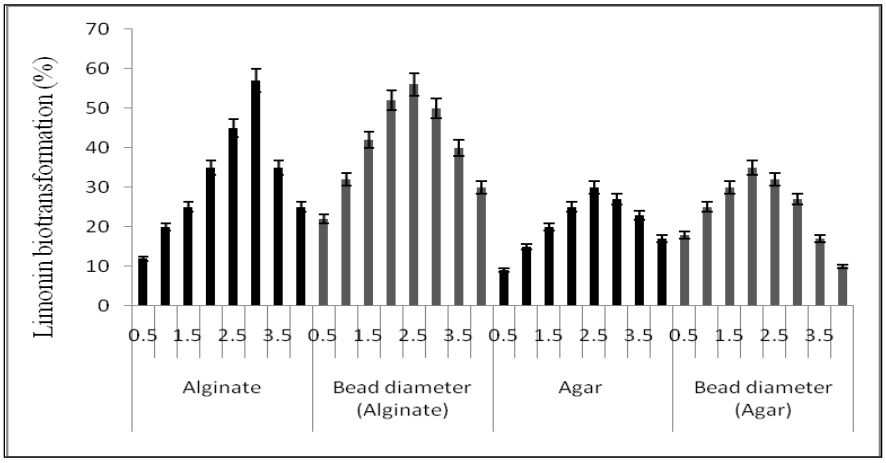


Fig. 2. Effect of alginate and agar concentration (%) with their respective bead size (mm) on limonin biotransformation with immobilized *P. putida* G7 cells.

3.2 Statistical analysis

The combined effect or the interrelationship of the parameters for limonin biotransformation response was observed with a face centered cube design of 2^3=8 + 6 center points and 6 (2x3) star points which lead to a total of 20 experiments. Based on the optimization of process parameters and the experimental results (obtained from CCD and regression analysis) (Table 2), the relationship between the limonin biotransformation and the significant parameters (alginate concentration, cell load and bead diameter) was established in the form of a quadratic polynomial equation. The equation for the model in terms of coded factors is as follows:

$$\textbf{Limonin biotransformation (R1)} = +64.43 + 0.70 * A + 2.56 * B + 1.40 * C - 2.60 * A * B - 1.07 * A * C - 0.37 * B * C - 0.28 * A2 - 3.42 * B2 - 1.60 * C2$$

Where, Y is the response value for limonin biotransformation (%), A-Alginate concentration, B-Cell Load, C-Bead diameter, R1-Limonin Biotransformation (%), R2-Stability (O.D. 600nm).

The analysis of variance (ANOVA) of the model for limonin biotransformation is presented in Table 3. The F value (14.97) of the model signifies the significance of the model and there was only 0.01% chance that the model F value must have occurred due to noise. Regression analysis results have revealed R^2 (coefficient of determination and the value more closer to 1 indicates the model fit of the experimental data) value of 0.9309, which signifies that the model was enabled to explain only 7% of the total variance. The adjusted R^2 (adjusted determination coefficient) value of 0.8687 was quite high which indicates the high reliability of the model and the quadratic polynomial equation. The values of "Prob>F" of the model far less than 0.05, indicates the significant and desirability of the model terms. The "Lack of Fit F-value" of 843.93 implies the Lack of Fit is significant. There is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. "Adeq Precision" measures the signal to noise ratio (a ratio greater than 4 is desirable) and the ratio of 12.862 indicates an adequate signal. A low value of CV (coefficient of variation) of

2.76% signifies a high degree of precision and a good deal of reliability of the experimental values. According to this model, the significant parameters A-B has shown the highest interaction effect, followed by A-C and B-C with the least interaction, for the maximal response of limonin biotransformation.

The experimental relationship between the stability of the beads (response) and the three significant parameters in the coded values is presented as:

$$\text{Stability (R2)} = +0.11 + 0.017 * A + 8.660E - 003 * B + 6.983E - 003 * C - 0.013 * A * B - 7.625E - 003 * A * C - 3.125E - 003 * B * C + 6.058E - 003 * A^2 + 3.407E - 003 * B^2 + 0.013 * C^2$$

Where, Y is the response value for limonin biotransformation (%), A-Alginate concentration, B-Cell Load, C-Bead diameter, R1-Limonin Biotransformation (%), R2-Stability (O.D. 600nm). This response is of great importance in estimating the bead scabrousness for their potential to withstand mechanical stress23. The analysis of variance (ANOVA) for this model is presented in Table 4.

The F value (14.97) of the model signifies the significance of the model and there was only 0.01% chance that the model F value must have occurred due to noise. Regression analysis results presenting the R^2 (coefficient of determination) and adjusted R^2 (adjusted determination coefficient) values of 0.9253 and 0.8581 respectively, was quite high which indicates the high reliability of the model and the quadratic polynomial equation. The values of "Prob>F" of the model far less than 0.05, indicates the significant and desirability of the model terms. The "Lack of Fit F-value" of 5.31 implies the Lack of Fit is significant and the corresponding 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. "Adeq Precision" ratio value of 12.683 indicates an adequate signal which signifies a high degree of precision with a good deal of reliability of the experimental values. According to this model, the significant variables A-B has shown the highest interaction effect, followed by A-C and B-C with the least interaction, for the maximal response of the high stability of the alginate beads.

Table 2. Response surface central composite design (CCD) and experimental limonin biotransformation.

Std. Order	Runs	A	B	C	Experimental (R1)	Predicted (R1)	Experimental (R2)	Predicted (R2)
1	10	1.00	40.00	1.50	49.7	50.42	0.07	0.072
2	8	3.00	40.00	1.50	61.8	59.17	0.16	0.15
3	11	1.00	50.00	1.50	62.7	61.49	0.13	0.12
4	13	3.00	50.00	1.50	60.1	59.83	0.138	0.15
5	4	1.00	40.00	2.50	56	56.12	0.12	0.11
6	15	3.00	40.00	2.50	59.5	60.57	0.15	0.15
7	18	1.00	50.00	2.50	63.2	65.69	0.138	0.14
8	9	3.00	50.00	2.50	60.6	59.74	0.145	0.14
9	2	0.32	45.00	2.00	63.8	62.47	0.089	0.094
10	17	3.68	45.00	2.00	63.3	64.83	0.15	0.15
11	1	2.00	36.59	2.00	50.1	50.45	0.092	0.10
12	14	2.00	53.41	2.00	59.2	59.05	0.132	0.13
13	3	2.00	45.00	1.16	55.6	57.54	0.126	0.13
14	19	2.00	45.00	2.84	64	62.25	0.15	0.15
15	16	2.00	45.00	2.00	64.5	64.43	0.11	0.11
16	7	2.00	45.00	2.00	64.4	64.43	0.11	0.11
17	20	2.00	45.00	2.00	64.5	64.43	0.11	0.11
18	12	2.00	45.00	2.00	64.4	64.43	0.1	0.11
19	6	2.00	45.00	2.00	64.3	64.43	0.11	0.11
20	5	2.00	45.00	2.00	64.5	64.43	0.11	0.11

Table 3. Analysis of variance (ANOVA) of the model for limonin biotransformation.

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	379.49	9	42.17	14.97	0.0001	significant
A-Alginate conc.	*6.69*	*1*	*6.69*	*2.38*	*0.1543*	
B-Cell load	*89.21*	*1*	*89.21*	*31.67*	*0.0002*	
C-Bead diameter	*26.79*	*1*	*26.79*	*9.51*	*0.0116*	
AB	*54.08*	*1*	*54.08*	*19.20*	0.0014	
AC	*9.24*	*1*	*9.24*	*3.28*	0.1001	
BC	*1.12*	*1*	*1.12*	*0.40*	0.5416	
A^2	*1.09*	*1*	*1.09*	*0.39*	0.5476	
B^2	*168.74*	*1*	*168.74*	*59.91*	<0.0001	
C^2	*36.94*	*1*	*36.94*	*13.12*	0.0047	
Residual	28.16	10	2.82			
Lack of Fit	*28.13*	*5*	*5.63*	*843.93*	*< 0.0001*	significant
Pure Error	*0.033*	*5*	*6.667E-003*			
Cor Total	407.66	19				

Table 4. Analysis of variance (ANOVA) of the model for stability.

Source	Sum of Squares	df	Mean Square	F -value	p- Value (Prob > F)	
Model	0.010	9	1.157E-003	13.76	0.0002	significant
A-Alginate conc.	*4.133E-003*	*1*	*4.133E-003*	*49.16*	*< 0.0001*	
B-Cell load	*1.024E-003*	*1*	*1.024E-003*	*12.18*	*0.0058*	
C-Bead diameter	*6.659E-004*	*1*	*6.659E-004*	*7.92*	*0.0183*	
AB	*1.378E-003*	*1*	*1.378E-003*	*16.39*	0.0023	
AC	*4.651E-004*	*1*	*4.651E-004*	*5.53*	0.0405	
BC	*7.812E-005*	*1*	*7.812E-005*	*0.93*	0.3578	
A^2	*5.289E-004*	*1*	*5.289E-004*	*6.29*	0.0310	
B^2	*1.672E-004*	*1*	*1.672E-004*	*1.99*	0.1888	
C^2	*2.288E-003*	*1*	*2.288E-003*	*27.21*	0.0004	
Residual	8.407E-004	10	8.407E-005			
Lack of Fit	*7.074E-004*	*5*	*1.415E-004*	*5.31*	*0.045*	significant
Pure Error	*1.333E-004*	*5*	*2.667E-005*			
Cor Total	0.011	19				

3.3 RSM analysis

The response model developed was further represented in the form of contour plots for a better understanding of the interaction among the three significant parameters and for the detection of the optimum level of each parameter for a maximum response. The contour plot showed the interaction of two independent parameters when the third parameter is fixed at zero (Fig. 3 a-c), which represents the limonin biotransformation response. Limonin biotransformation increases moderately with increasing alginate concentration from 2% to 3% (w/v), with a high increase in cell load results in highly biotransformation (Fig. 3a). Fig. 3b demonstrates that response increases moderately with an increase in bead diameter and alginate concentration. An increased response was observed to increase in cell load, and bead diameter (Fig. 3c). The increase in limonin biotransformation when alginate concentration is increased (2-3%) can be due to the facilitation of mass transfer rate of the substrate and product formed[24-27]. The increase in response to an increase in bead size and cell load can be attributed with effective utilization of the total cell load for limonin bioconversion, within an increased bead space which had protected the reaction from the unfavorable external pH of the juice.

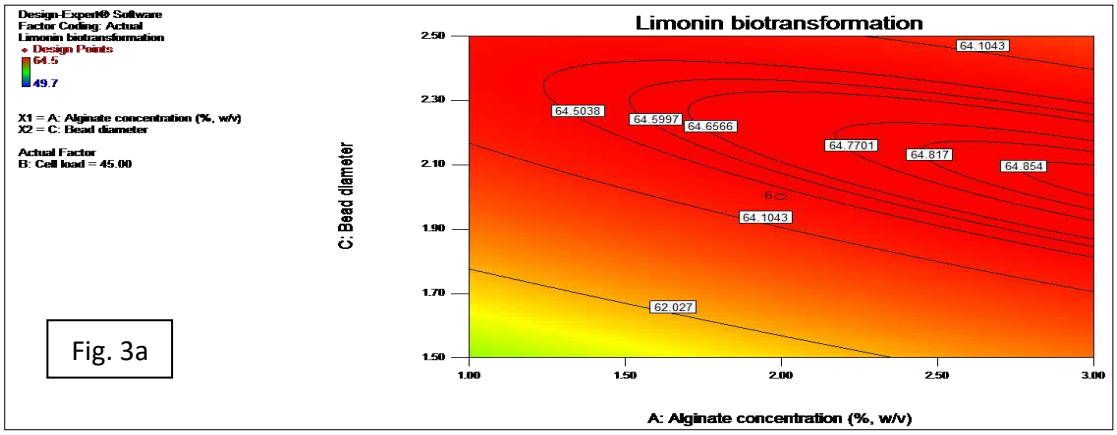


Fig. 3a

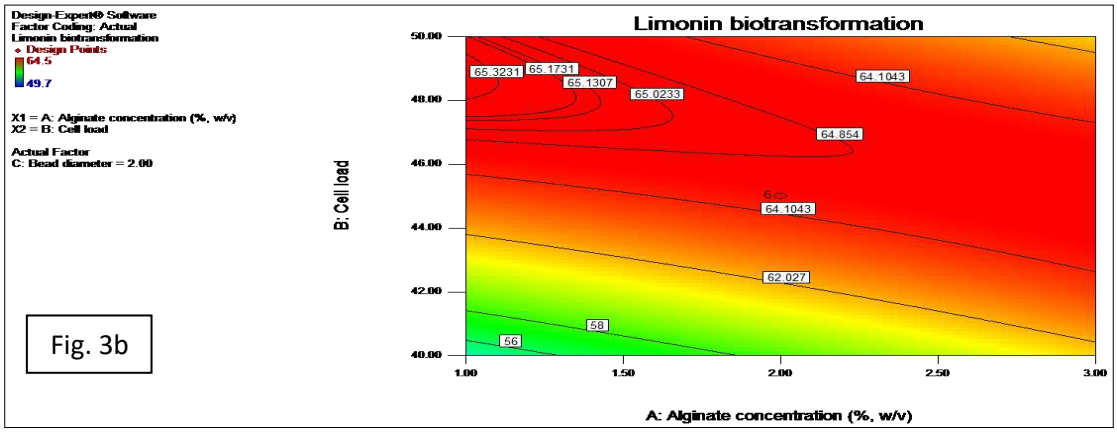


Fig. 3b

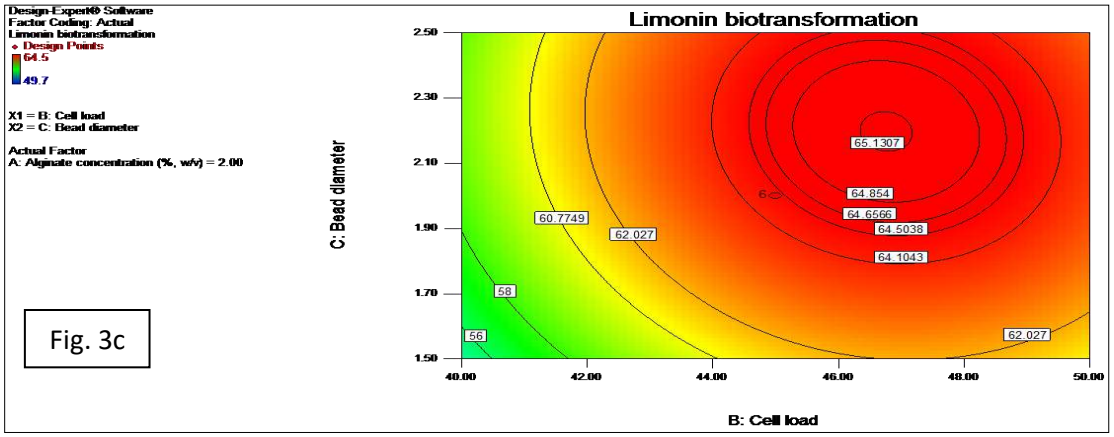
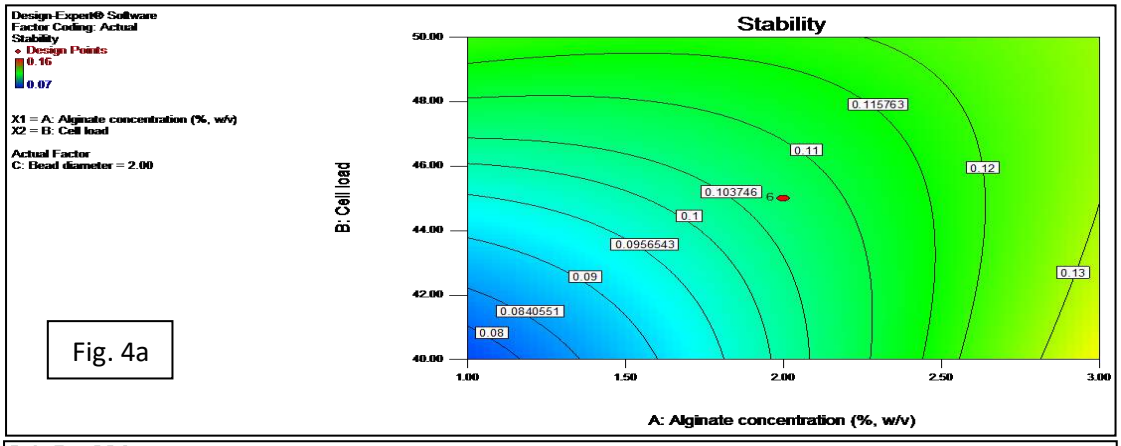
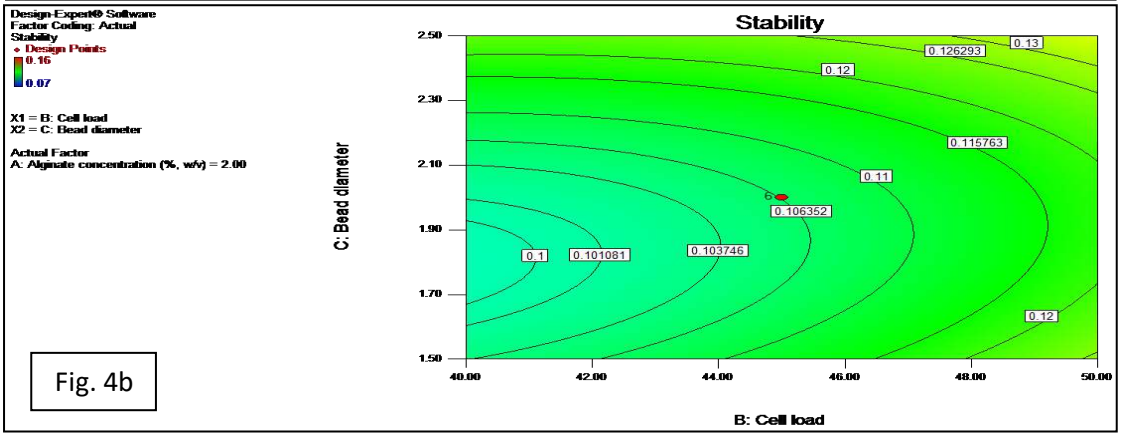


Fig. 3. Contour plots of limonin biotransformation which depicts the interaction among a. alginate concentration (%) and bead diameter (mm), b. alginate concentration (%) and cell load (mg/ml) c. bead diameter and cell load (mg/ml).

The contours for stability with regard to the significant parameters of alginate concentration, cell load, and bead diameter are presented in Fig. 4a-c. Fig. 4a shows that higher stability could be obtained at a moderate alginate concentration and low cell load. Fig. 4b dictates that stability increases with an increased bead diameter and at a moderate alginate concentration. Low cell load and a moderate bead diameter direct towards an increased response for stability (Fig. 4c). Moderate concentration of alginate supports for stable beads as low concentrations causes cell leakage due to flimsy beads and high concentrations of alginate direct the beads to a brittle nature[24,28,29].





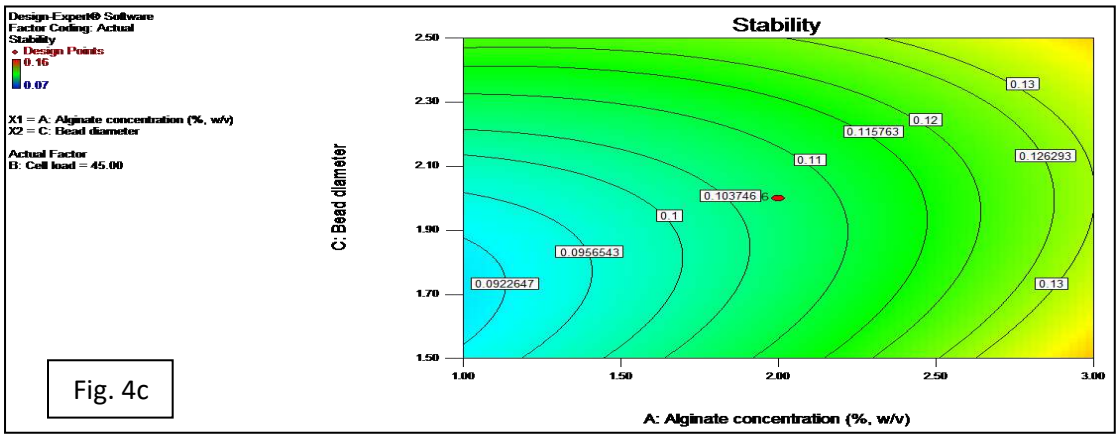


Fig. 4. Contour plots of stability which depicts the interaction among a. alginate concentration (%) and cell load (mg/ml), b. bead diameter (mm) and cell load (mg/ml), c. alginate concentration (%) and bead diameter (mm).

3.4 Validation of the model

The experimental model was validated by considering both activity and stability for a high limonin biotransformation and low OD600 nm responses, with the help of the regression equation. The experimental reactions (100ml Erlenmeyer flask containing 50ml citrus juice) with immobilized cells, for the maximum response, included an alginate concentration of 2%, a cell load of 47.2g/l, and a bead diameter of 2.1mm. The predicted values for the maximum response of limonin biotransformation and stability giving these conditions were 65.1% and 0.094 respectively. The experiments were conducted in triplicates and maximum response to all the optimized conditions were 65.8% and 0.97 OD.

3.5 Re-usability of immobilized cell

The re-usability of the immobilized cells *P. putida* G7 was compared with free cells (20 beads, equivalent to 47mg/ml cells and free cells - 47mg/ml cells), for the evaluation of limonin biotransformation under the optimized parameters, at the original pH of citrus juice (4 pH) and at 35°C.

The immobilized bacterial cells and free cells were evaluated for their biotransformation potential in successive cycles. After each cycle, the alginate beads were filtered and the free cells were collected by centrifugation, washed with saline and phosphate buffer and were then reused for limonin biotransformation in the next set of citrus juice. All the reaction conditions were kept constant for every batch cycle. The biotransformation reaction was carried out for 3 hours and the bioconversion during each cycle by immobilized and free cells is presented in Fig. 5. The results depict a faster bioconversion and high reusability (till the 8th cycle) of immobilized cells as compared to free cells (till the 5th cycle). This high performance of the immobilized cells indicates towards the higher rate of limonin biotransformation by *P. putida* G7, which indicate that the immobilization matrix has protected the cells from the external environment (low pH and other cell activity hampering essential oils or inhibitors in the juice), for a better performance. The optimization of immobilization parameters for immobilizing bacterial cells to get an enhanced response for limonin biotransformation of 65% in a time span of 3 hours has an advantage over the other immobilizing matrices used so far (which shows a delayed limonin biotransformation in up to 50-60 hrs)[29].

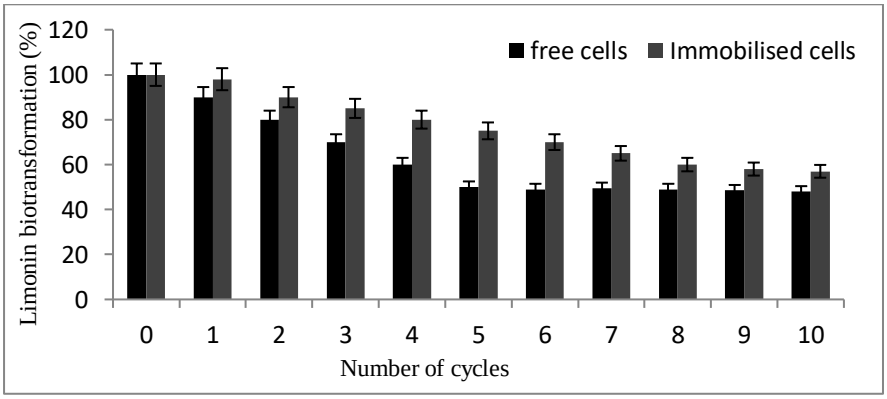


Fig. 5. Re-usability of free and alginate immobilized *P. putida* G7 cells for limonin biotransformation.

4. Conclusion

The employment of immobilized *P. putida* G7 cells for an enhanced limonin biotransformation in a very short span of time, under optimized conditions; prove to be a promising technology for debittering citrus juices. This study indicates the significance and essentiality of statistical tools like RSM, for optimization of process parameters in order to improve the respective response.

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